Controlled trial of whole body protein synthesis and plasma amino acid concentrations in yearling horses fed graded amounts of lysine

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17   **Abstract**

18   Lysine has been reported as the first limiting amino acid in typical equine diets. Indicator
19   amino acid oxidation (IAAO) has become the standard method for determining amino acid
20   requirements in other species, but prior to this study has not been used to determine equine
21   requirements. The aim of this study was to evaluate whole body protein synthesis and plasma and
22   muscle amino acid concentrations in response to graded levels of lysine intake in yearling horses.
23   Six Thoroughbred colts (358 ± 5 kg) were fed each of six treatment lysine intakes ranging from 76
24   to 136 mg/kg body weight/day. Blood samples were taken before and 90 min after the morning
25   concentrate meal. Gluteal muscle biopsies were taken ~100 min after the morning concentrate meal.
26   The next day, whole body phenylalanine kinetics were determined using a 2 h primed, constant
27   infusion of [^{13}C] sodium bicarbonate followed by a 6 h primed, constant infusion of [1- ^{13}C]
28   phenylalanine. Plasma lysine concentrations increased linearly ($P < 0.05$) at both the 0 and 90 min
29   time points with increasing lysine intakes. Free muscle asparagine, aspartate, arginine, glutamine,
30   lysine, taurine and tryptophan concentrations responded quadratically to lysine intake ($P < 0.05$).
31   Phenylalanine kinetics did not differ between treatment intakes ($P > 0.10$). A broken line analysis of
32   lysine intake and phenylalanine oxidation failed to yield a breakpoint from which to determine a
33   lysine requirement. These diets may have been limiting in an amino acid other than lysine,
34   underscoring the lack of data concerning amino acid requirements and bioavailability data in the
35   horse.

36

37   *Keywords:* Equine; Amino acid; Lysine; Indicator amino acid oxidation; Protein synthesis

38 **Introduction**

39   Compared to human beings and pigs, relatively little work has been done to elucidate amino
40 acid requirements for horses. Currently, the NRC (2007) lists only a crude protein requirement and
41 a lysine requirement in the requirement tables and software. Identifying amino acid requirements
42 for various physiological states (NRC, 2012) has helped the pig industry to minimize nitrogenous
43 waste (Panetta et al., 2006) without compromising pig growth (Tuitoek et al., 1997). The
44 environmental impacts of excess nitrogen output from equine operations have been reviewed by
45 Bott et al. (2015). Other consequences of overfeeding protein to horses could be compromised
46 respiratory health from increased ammonia output (Whittaker et al., 2009), disrupted acid-base
47 balance (Graham-Thiers and Kronfeld, 2005b) and decreased bone mineralization from increased
48 calcium excretion through urine (Glade et al., 1985). Determining amino acid requirements in
49 horses will allow diet formulation to more closely match equine needs.

51   Lysine is a common limiting amino acid for many species (Metges et al., 2005; Tujioka et
52 al., 2005; Tome and Bos, 2007). It has been found to be limiting in some diets for growing horses
53 (Breuer and Golden, 1971; Potter and Huchton, 1975; Ott et al., 1981; Fisher et al., 1989; Graham
54 et al., 1994). However, these studies in horses generally were not designed to measure specific
55 lysine requirements and they did not use isonitrogenous treatments, but rather used treatments with
56 different protein sources and/or did not include many levels of lysine intake. The NRC (2007)
57 applied broken line analysis to seven studies that reported diet composition, intake and nitrogen
58 retention to estimate a lysine requirement at 4.3% of the crude protein requirement (NRC, 2007).
59 This lysine requirement as a percentage of crude protein is in line with other species (Ball et al.,
60 2007), but has not been confirmed experimentally in horses. The current NRC (2007)
61 recommendation for lysine for yearling horses is 113 mg/kg body weight (BW)/day.

62

63 Isotopic methods have been used in other species to determine amino acid requirements
64 (Bertolo et al., 2005; Kurpad and Thomas, 2011). These methods are based on the concept that
65 amino acids that cannot be used for protein synthesis are oxidized to CO₂. The production of CO₂
66 from amino acid metabolism can be determined from an infusion of a 1-¹³C-labeled amino acid and
67 measuring ¹³CO₂ expelled in the breath. Using an isotopic essential amino acid other than the amino
68 acid being tested as an indicator of amino acid metabolism is known as the indicator amino acid
69 oxidation (IAAO) method (Brunton et al., 1998; Elango et al., 2008). As the dietary intake of a test
70 amino acid, such as lysine, increases, less of the indicator or 1-¹³C-labeled amino acid is oxidized
71 until a plateau is reached. The intersection of this decline and plateau of the rate of ¹³CO₂ release
72 from the indicator amino acid defines the requirement of the test amino acid. The isotope infusion
73 technique necessary to determine requirements via the IAAO method has been adapted to horses
74 and validated (Urschel et al., 2012). In addition to calculating amino acid requirements, the isotopic
75 data can also yield estimates of rates of whole body protein synthesis and breakdown (Waterlow et
76 al., 1978).

77
78 This study investigated the responses of whole body protein synthesis in addition to plasma
79 and tissue amino acid concentrations in yearling horses receiving graded levels of lysine. The lysine
80 levels included levels both above and below the current NRC (2007) recommendations. We
81 hypothesized that a breakpoint would be seen in our phenylalanine kinetics data denoting a lysine
82 requirement for yearling horses.

83
84 **Materials and methods**

85 *Animals, housing, and feeding*

86 All procedures used in this study were approved by the University of Kentucky Institutional
87 Animal Care and Use Committee (IACUC 2008-0385). Six yearling Thoroughbred colts were
88 obtained from University of Kentucky Maine Chance Farm for use in this study in May 2011. The

IAAO method has successfully been used to determine amino acid requirements in other species using five to six subjects (Bertolo et al., 2005; Elango et al., 2007; Levesque et al., 2011). A sample size calculation confirmed that six horses were appropriate (PS Power and Sample Size Calculations Program, version 2.1.3.1).

The adaptation diet consisted of hay cubes and a half and half mix of the concentrates used in the treatment diets. The 2 week adaptation period to diet and housing was followed by six 1 week treatment periods. Yearlings were housed in individual dry lot pens and all sampling procedures were conducted in individual sawdust bedded stalls (3.7 m x 3.7 m) except for muscle biopsies, which were collected from horses in stocks. A livestock scale (TI-500, Transcell Technology) was used to obtain daily body weights.

Diets were designed to meet or exceed the 2007 Nutrient Requirements of Horses recommendation, with the exception of lysine (NRC, 2007). Two isonitrogenous and isocaloric versions of the same concentrate pellets were formulated for this study (Tables 1 and 2). The only differences between the two concentrates were that one contained free lysine, and the other an isonitrogenous amount of free glycine and a small amount of rolled oats to account for the difference in weight of the added glycine versus lysine. By mixing the two concentrates at different ratios and feeding timothy hay cubes at the same rate for all treatments, six treatment diets were created with lysine intakes of 76, 90, 104, 118, 127 and 136 mg/kg BW/day. Three of the lysine intakes were above the estimated lysine requirement of 113 mg/kg BW/day and three of the intakes were below. Glycine was selected because it is a dispensable amino acid and not metabolically related to lysine. The concentrate was provided at 1.11% of BW/day with timothy hay cubes at 1.37% of BW/day (Table 1). Timothy was used as the forage component of the diets because of its relatively low lysine content (Woodward et al., 2011). Concentrate and hay cubes were fed twice daily (0700 and 1500 h) with water and salt available at all times. Feed was collected throughout the

115 study and four samples of each feedstuff were sent to Dairy One Cooperative for proximate analysis
116 at the conclusion of the study. Weekly feed samples were analyzed for amino acid content as
117 described below.

118

119 *Study design and procedures*

120 Horses were studied in a group of two and a group of four with similar birth dates within
121 each group to facilitate sample collection. Splitting the horses into groups allowed all the horses to
122 be studied at similar ages and further minimize variation. Diets were fed in a random order within
123 the groups, with the stipulation that no two horses were on the same treatment diet at the same time.
124 Treatment assignments were made after horses were selected for the study using a random number
125 generator, such that each treatment was represented in each period and each horse only received
126 each treatment a single time,. Researchers were not blinded to the treatments to provide multiple
127 checks to ensure that each horse received the correct diet during each period. Each horse received
128 each diet as in a 6 x 6 Latin square design.

129

130 Following the initial adaptation, treatment diets were fed for a period of 7 days each. This
131 length of adaptation was chosen because less than 2 days of adaptation has been found to be
132 sufficient for human beings (Elango et al., 2009) and pigs (Moehn et al., 2004), but the effect of
133 adaptation time on IAAO analyses has not yet been ascertained in horses. On day 6 of treatment,
134 blood samples (10 mL) were collected by venipuncture immediately before and 90 min after the
135 morning concentrate meal into an evacuated glass tube containing a heparin to measure plasma
136 glucose, insulin, urea nitrogen and amino acid concentrations. Horses were then sedated with 100
137 mg/mL xylazine (AnaSed Lloyd Laboratories) administered IV at a dose of 0.5 mg/kg BW. Muscle
138 biopsies (~500 mg) were taken after sedation took effect (~100 min post-feeding) from the gluteus
139 medius at a depth of ~6 cm with a Bergstrom needle (Lindholm and Piehl, 1974). The muscle was
140 immediately frozen in liquid nitrogen. While the horses were still sedated, both jugular veins were

141 catheterized, one side for isotope infusion and the other for blood sampling (Urschel et al., 2012) in
142 preparation for the following day's procedures.

143

144 On day 7 of treatment, whole body phenylalanine kinetics were measured as described
145 previously (Mastellar et al., 2016b), with the exception that the infusion of [1-¹³C] phenylalanine
146 continued for 6 h instead of only 4 h. Upon completion of the isotope infusion procedures, catheters
147 were removed and the yearlings were allocated to their next treatment, beginning with their
148 afternoon concentrate meal for that day. Samples were collected during the months of May, June
149 and July of 2011. Yearlings were returned to University of Kentucky Maine Chance Farm research
150 herd at the end of the study.

151

152 *Analysis of samples*

153 *Blood samples* - Heparinized vacutainers (Vacutainer; Becton-Dickinson) were used for
154 blood collection. Samples were inverted and then promptly centrifuged at 1500 g for 10 min at 4 °C.
155 Plasma was removed and frozen at -20 °C until the time of analysis.

156

157 *Amino acids* - Plasma free, muscle free and total feed amino acid concentrations were
158 measured using reverse phase high performance liquid chromatography (HPLC; 3.9 x 300 mm
159 PICO-TAG reverse phase column; Waters) of phenylisothiocyanate derivatives, as previously
160 described (Urschel et al., 2011). Muscle samples were freeze dried (Miller-Graber et al., 1990) and
161 homogenized in 6 µL/µg of 0.4 mM nor-leucine made in 0.1 N HCl prior to deproteination. Both
162 muscle homogenates and plasma samples were deproteinated by centrifugation at 14,000 g for 30
163 min at 4 °C using 10 kDa cutoff centrifugation filters (Urschel et al., 2011). Six samples of each
164 feedstuff collected throughout the study were used for amino acid analyses (AOAC, 2005), with
165 each sample analyzed in duplicate.

166

167 *Plasma metabolites* - A YSI 2300 STAT Plus Glucose and Lactate Analyzer (YSI) was used

168 to determine plasma glucose concentrations enzymatically. The intra-assay variation was 1.5% with
169 all samples being run in a single batch. As previously described (Urschel et al., 2007), a
170 colorimetric spectrophotometric assay was used to determine plasma urea nitrogen, with an intra-
171 assay variation of 3.5% and an inter-assay variation of < 10%. A Coat-A-Count RIA kit (Siemens
172 Healthcare Diagnostics), previously validated for use in horses (Reimers et al., 1982), was used to
173 determine plasma insulin concentrations.

174
175 *Isotope enrichment* - Immediately after sampling, the amount of $^{13}\text{CO}_2$ relative to $^{12}\text{CO}_2$ in
176 breath samples collected throughout the isotope infusions was determined using an infrared isotope
177 analyzer (IRIS-2; Wagner Analysen Technik Vetriebs GmbH). Phenylalanine isotopic enrichment,
178 representing the relative concentrations of [1- ^{13}C] phenylalanine to unlabeled phenylalanine, in
179 plasma samples was measured by Metabolic Solutions using a previously described method
180 (Matthews et al., 1990).

182 *Calculations*

183 *Whole body phenylalanine kinetics* - The average enrichment at plateau was determined for
184 blood and breath sample enrichments, where a plateau was defined as at least three points with a
185 slope that was not different from 0 ($P > 0.05$) using linear regression analyses (GraphPad Prism 4
186 Software). Whole body phenylalanine flux, oxidation, non-oxidative disposal and phenylalanine
187 balance were calculated as previously described (Tanner et al., 2014). Intake was adjusted for
188 estimated prececal digestibilities of 40 and 70% for forage and concentrate, respectively, based on a
189 review by Martin-Rosset and Tisserand (2004), and a splanchnic extraction of 26.5% (Mastellar et
190 al., 2016a) to more accurately reflect the absorbed phenylalanine entering general circulation. Non-
191 oxidative phenylalanine disposal was used as an indicator of whole body protein synthesis.

192 Conversion of phenylalanine to tyrosine was considered negligible and, if any occurred, the intake
193 of phenylalanine and tyrosine was the same across treatments.

194

195 *Statistical analysis*

196 Data were analyzed using the MIXED procedure in SAS version 9.1.3 (SAS Institute),
197 unless otherwise noted. Differences were considered to be significant at $P < 0.05$. One-way analysis
198 of variance (ANOVA) with treatment as the fixed effect and horse as the random effect, including
199 analysis for linear, quadratic and cubic orthogonal contrasts, was used to evaluate phenylalanine
200 kinetics parameters, pre-feeding and post-feeding plasma glucose, insulin, urea and amino acid
201 concentrations, and muscle amino acid concentrations. Period and group were originally included in
202 the statistical model, but were found not to be significant. Given that the treatment levels of lysine
203 were unequally spaced, the coefficients for the orthogonal polynomial contrasts were generated
204 using SAS PROC IML. A repeated measures analysis, with treatment, time and time x treatment
205 interaction as fixed effects, and horse nested within treatment as the random effect, was used to
206 analyze plasma amino acid, urea, glucose and insulin concentration data. The autoregressive
207 variance-covariance matrix was used to analyze the repeated measures data. In all cases, when fixed
208 effects were significant, the means were separated using the Tukey-adjusted pdiff option. A broken
209 line analysis, as described by Robbins et al. (2005), using the NLIN procedure in SAS was
210 performed for lysine intake and phenylalanine oxidation. Starting estimates of breakpoint and line
211 slope were determined using a method previously described by Fadel (2004). This method of
212 broken line analysis has been applied to IAAO data for the determination of amino acid
213 requirements in pigs (Levesque et al., 2011).

214

215 **Results**

216 All horses remained healthy throughout the study, completed all study treatments and were
217 included in all analyses per protocol. Horses were an average of 400 ± 5 days old and had a BW of

218 358 ± 5 kg on isotope infusion study days. Average daily gain for the yearlings while on treatment
219 diets was 0.25 kg/day.

220

221 *Amino acid intakes*

222 On the basis of muscle profiles, amino acid intakes exceeded NRC requirements (NRC,
223 2007), with the exceptions of histidine and threonine (Table 3). Lysine was also below NRC
224 requirements (NRC, 2007) for three of the treatments, as dictated by the study design.

225

226 *Plasma amino acid concentrations*

227 Compared with baseline values (0 min) (Table 4), plasma amino acid concentrations were
228 greater at 90 min post-feeding ($P < 0.001$) (Table 5). Plasma lysine and glycine concentrations
229 increased linearly ($P < 0.05$) within the 0 and 90 min time points with increasing lysine and glycine
230 intakes, respectively (Tables 4 and 5).

231

232 *Plasma metabolite concentrations*

233 Plasma urea nitrogen, glucose, and insulin concentrations (Tables 4 and 5) were greater
234 post-feeding (Table 5) than baseline values (Table 4; $P < 0.01$), but were not affected by treatment
235 ($P > 0.05$). The interactions between time and treatment were also not significant ($P > 0.05$), nor
236 were linear, quadratic or cubic effects ($P > 0.05$).

237

238 *Muscle free amino acid concentrations*

239 Gluteus medius muscle free amino acid concentrations (Table 6) did not respond to
240 changing lysine intakes in the same manner as plasma amino acid concentrations (Tables 4 and 5).
241 Free asparagine, aspartate, arginine, glutamine, lysine, taurine and tryptophan concentrations
242 responded quadratically to lysine intake ($P < 0.05$). Free tyrosine concentrations responded
243 cubically to lysine intake ($P = 0.04$).

244

245 *Phenylalanine kinetics*

246 Phenylalanine and tyrosine intakes were equivalent for all treatments. Phenylalanine flux,
247 release from protein breakdown, oxidation, non-oxidative disposal and balance did not differ
248 between treatments ($P > 0.10$; Table 7). Linear, quadratic and cubic orthogonal contrasts on
249 phenylalanine kinetic parameters were also not significant ($P > 0.05$). A broken line analysis of
250 lysine intake and phenylalanine oxidation failed to yield a breakpoint from which to determine a
251 lysine requirement for yearling Thoroughbred colts (Fig. 1).

252

253 **Discussion**

254 This study underscores the limitations in our current knowledge regarding equine amino
255 acid requirements and the need for further investigation into amino acids other than lysine that were
256 potentially limiting in our treatment diets. We were unable to determine a breakpoint using the
257 phenylalanine oxidation data (Fig. 1). Rates of non-oxidative phenylalanine disposal (Table 7) were
258 not affected by treatment, but were less than those measured in weanlings (Tanner et al., 2014;
259 Mastellar et al., 2016b) and only slightly greater than those measured in adult mares (Mastellar et
260 al., 2016b). Phenylalanine balance was positive (Table 7), indicating that the yearlings were
261 accreting protein. However, as this is the first time whole body protein synthesis was measured in
262 yearling horses using this method, there is no data to compare these values with to determine if
263 protein accretion was submaximal for this age of horse. Maximal whole body protein synthesis for
264 yearlings would be expected to be only slightly higher than maximal for mature horses, since the
265 average daily gain of yearlings is relatively small (Stanier et al., 2004).

266

267 The plasma metabolite concentrations also did not indicate a requirement (Tables 4 and 5).
268 A previous study by Ohta et al. (2007) found a broken line in plasma lysine concentrations when
269 feeding graded amounts of lysine. However, in the present study, we found a linear increase in 90

270 min post-feeding plasma lysine concentrations with increasing lysine intake (Table 5). Additionally,
271 there was no decrease in plasma threonine or methionine concentrations with increasing lysine
272 intake, similar to that found by Malesky et al. (2013). Our plasma urea measurements also did not
273 differ due to treatment as would be expected if an amino acid other than lysine was limiting in the
274 basal diet. However, the timing of our blood sampling may not have been optimal to detect changes
275 in plasma urea concentrations due to treatment, since another study found significant differences in
276 plasma urea concentrations due to amino acid supplementation in horses sampled 3-4 h after meal
277 feeding (Graham-Thiers and Kronfeld, 2005a).

278

279 Our data suggest that the lysine requirement of horses could be below the lowest level of
280 lysine intake in the study (76 mg/kg BW/day). However, this is unlikely given the suggested
281 requirements indicated in previous studies (Breuer and Golden, 1971; Fisher et al., 1989; NRC,
282 2007; Ohta et al., 2007). Another possibility would be that our IAAO data were unable to yield a
283 lysine requirement for yearling horses receiving these diets (Table 1). An amino acid other than
284 lysine that is equally limiting across our treatments diets could explain why a requirement could not
285 be determined. This limitation would also explain our relatively poor growth rate over the course of
286 the study.

287

288 Protein synthesis rates would not differ if all the diets were equally limiting in some amino
289 acid other than lysine. Protein synthesis rates affect amino acid oxidation, plasma amino acid
290 concentrations and plasma urea concentrations. Since there is limited data regarding amino acid
291 requirements in horses, developing diets to contain adequate amounts of particular amino acids
292 other than the test amino acid is inherently difficult. Candidates for universally limiting amino acids
293 in the treatment diets include threonine and histidine (Table 3). The plasma urea data may be
294 evidence of a universally limiting amino acid across treatments, since these values were relatively

295 high when compared to other data reported in the literature (Graham-Thiers and Kronfeld, 2005a, b;
296 Winsco et al., 2011).

297

298   Using ratios for muscle tissue amino acids and the lysine requirement estimate provided by
299 the NRC (2007), the threonine requirement estimate could be 70 mg/kg BW/day for yearling horses
300 (Bryden, 1991). Using ratios from other researchers that reported total equine muscle amino acids,
301 the requirement would be ~60 mg/kg BW/day (Badiani et al., 1997; Lorenzo and Pateiro, 2013).
302 Using some of these estimates, our rations may have been deficient in threonine, which would
303 explain the lack of response to lysine intake. Threonine digestibility has been shown to vary greatly
304 in pigs according to feedstuff (Myrie et al., 2008).

305

306   If threonine was limiting, as the intake data suggests, it would be expected that threonine
307 plasma concentrations would have been lower than is typical for equine plasma. The average across
308 treatments in our study was 131 $\mu\text{mol/L}$ threonine before feeding (Table 4). However, when
309 compared to the values given in previous studies (Fisher et al., 1989; Hackl et al., 2006; Tanner et
310 al., 2014) it does not appear to be low, which contradicts the idea that threonine could have been
311 limiting across all treatment diets. Given the lack of data in the literature specifically on yearling
312 plasma amino acid concentrations and differences in analysis methods, it is difficult to determine
313 whether the plasma threonine concentrations in our study were below the normal range.

314

315   Using the muscle amino acid ratios, histidine also appears to be a limiting amino acid in our
316 diets. Histidine as a potentially limiting amino acid in equine diets has previously been discussed by
317 Tanner et al. (2014). More research is needed to determine the adequacy and bioavailability of the
318 amino acids provided in the treatment diets. Future research should endeavor to supply potential
319 indispensable amino acids above estimated requirements.

320

321   Although we were not able to determine a lysine requirement in this study, use of the IAAO
322 method for future research into amino acid requirements for horses should not be discouraged.
323 Phenylalanine kinetics were still able to distinguish between protein synthesis rates in weanling
324 horses fed two different levels of protein (Tanner et al., 2014). This method has also been
325 successfully used to determine lysine requirements in pigs (Bertolo et al., 2005) and human beings
326 (Elango et al., 2007). Future studies in horses should take care to supplement diets with
327 indispensable amino acids other than the test amino acid.

328

329   Our investigations found comparable gluteus medius free amino acid concentrations to those
330 previously reported from freeze dried muscle (Miller-Graber et al., 1990). Muscle tissue free amino
331 acids have been studied in the horse in response to exercise (Graham-Thiers et al., 2012), growth
332 (Manso Filho et al., 2009) and feeding (Urschel et al., 2011). However, to our knowledge, this is the
333 first time free tissue amino acids have been studied in response to supplementation of a single
334 amino acid with isonitrogenous treatments in horses.

335

336   The underlying reasons for the differences in gluteus medius free amino acid concentrations
337 between treatments are not entirely clear and more research is certainly warranted. Amino acid
338 transporters are regulated by dietary lysine in the intestine (Wang et al., 2012) and other tissues
339 (Humphrey et al., 2006). It is possible that lysine transporters could be upregulated at the lower
340 lysine intakes, resulting in greater levels of lysine in the muscle at the lower levels of lysine intake.
341 However, more research would need to be conducted to confirm this mechanism.

342

343 **Conclusions**

344   Supplementation of a single amino acid can affect the metabolism of amino acids at the
345 tissue level, even if whole body protein synthesis is not affected. Our inability to determine a lysine
346 requirement utilizing the IAAO method with these test diets underscores the paucity of data

347 concerning amino acid requirement, adequacy and bioavailability data in the horse. Equine amino
348 acid requirements are an area in need of further research.

349

350 **Conflict of interest statement**

351 None of the authors of this paper have a financial or personal relationship with other people
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353

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364

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Table 1

As-fed nutrient composition of feeds (mean $\pm$ standard error) used in creating treatments with six levels of lysine intake for a controlled trial of yearling horses.

	Timothy hay cubes ^a	Low lysine concentrate ^b	High lysine concentrate ^c
Overall nutrient composition			
Dry matter (%)	91.2 $\pm$ 0.2	89.3 $\pm$ 0.1	89.6 $\pm$ 0.5
DE (Mcal/kg) ^c	1.69 $\pm$ 0.11	2.97 $\pm$ 0.02	3.06 $\pm$ 0.06
Crude protein (%)	7.1 $\pm$ 0.1	13.9 $\pm$ 0.1	13.7 $\pm$ 0.3
Lignin (%)	4.9 $\pm$ 0.1	0.8 $\pm$ 0.3	0.9 $\pm$ 0.1
ADF (%)	39.4 $\pm$ 0.6	10.1 $\pm$ 0.5	9.3 $\pm$ 1.1
NDF (%)	60.4 $\pm$ 0.5	19.9 $\pm$ 0.7	17.5 $\pm$ 2.0
Water soluble carbohydrates (%)	10.9 $\pm$ 0.3	3.9 $\pm$ 0.1	4.1 $\pm$ 0.1
Ethanol soluble carbohydrates (%)	5.6 $\pm$ 0.2	2.3 $\pm$ 0.8	2.8 $\pm$ 0.3
Starch (%)	1.0 $\pm$ 0.1	32.3 $\pm$ 0.4	33.4 $\pm$ 1.9
Crude fat (%)	1.6 $\pm$ 0.1	6.7 $\pm$ 0.1	7.1 $\pm$ 0.1
Calcium (%)	0.37 $\pm$ 0.01	1.86 $\pm$ 0.10	1.94 $\pm$ 0.17
Phosphorus (%)	0.20 $\pm$ 0.01	0.30 $\pm$ 0.01	0.32 $\pm$ 0.02
Iron (mg/kg)	149 $\pm$ 6	175 $\pm$ 9	215 $\pm$ 61
Zinc (mg/kg)	101 $\pm$ 16	114 $\pm$ 6	126 $\pm$ 34
Amino acid composition (g/100 g feed)			
Alanine (%)	0.34 $\pm$ 0.03	0.62 $\pm$ 0.02	0.61 $\pm$ 0.01
Arginine (%)	0.35 $\pm$ 0.04	0.61 $\pm$ 0.03	0.60 $\pm$ 0.01
Aspartate + Asparagine (%)	0.51 $\pm$ 0.04	0.61 $\pm$ 0.06	0.53 $\pm$ 0.05
Glutamate + Glutamine (%)	0.64 $\pm$ 0.07	2.50 $\pm$ 0.09	2.57 $\pm$ 0.08
Glycine (%)	0.25 $\pm$ 0.02	1.06 $\pm$ 0.02	0.40 $\pm$ 0.01
Histidine (%)	0.05 $\pm$ 0.01	0.16 $\pm$ 0.01	0.16 $\pm$ 0.01
Isoleucine (%)	0.28 $\pm$ 0.03	0.51 $\pm$ 0.02	0.47 $\pm$ 0.02
Leucine (%)	0.47 $\pm$ 0.04	1.19 $\pm$ 0.02	1.19 $\pm$ 0.02
Lysine (%)	0.25 $\pm$ 0.02	0.37 $\pm$ 0.02	0.92 $\pm$ 0.05
Methionine (%)	0.02 $\pm$ 0.01	0.59 $\pm$ 0.06	0.70 $\pm$ 0.05
Phenylalanine (%)	0.32 $\pm$ 0.02	0.60 $\pm$ 0.01	0.60 $\pm$ 0.02
Proline (%)	0.36 $\pm$ 0.03	0.98 $\pm$ 0.03	0.98 $\pm$ 0.01
Serine (%)	0.19 $\pm$ 0.01	0.32 $\pm$ 0.04	0.45 $\pm$ 0.03
Threonine (%)	0.18 $\pm$ 0.01	0.38 $\pm$ 0.01	0.38 $\pm$ 0.01
Tyrosine (%)	0.14 $\pm$ 0.02	0.39 $\pm$ 0.03	0.39 $\pm$ 0.02
Valine (%)	0.35 $\pm$ 0.02	0.56 $\pm$ 0.02	0.53 $\pm$ 0.01

^a Timothy Balance Cube (Ontario Dehy).

^b Mixed for this experiment by Buckeye Nutrition; for ingredient composition see Table 2.

^c Diets were top-dressed with 0.43 mL/kg body weight/day canola oil to increase the DE of the diets.

580 **Table 2**
 581 Composition of high and low lysine concentrates for a controlled trial of yearling horses. ^a
 582

Ingredient	Amount (%)	
	Low lysine concentrate	High lysine concentrate
Wheat	46.558	46.551
Soy hulls	16.251	16.264
Oatmill byproduct	14.109	14.105
Corn gluten	7.221	7.22
Calcium carbonate	4.95	4.949
Soybean oil	4.967	4.966
Cane molasses	2.458	2.456
Corn distiller's dry grain	1.264	1.262
DL-Methionine	0.833	0.833
Glycine	0.669	-
Lysine	-	0.846
Rolled oats	0.171	-
Threonine	0.168	0.168
Gro 'N Win ^b	0.13	0.13
Selenium premix	0.093	0.093
Zinc sulfate	0.029	0.03
Magnesium oxide	0.027	0.027
Monocalcium phosphate (21%)	0.028	0.028
Dyna K ^c	0.028	0.028
Dynamate ^d	0.028	0.028
Copper II Sulfate	0.018	0.018

583
 584 ^a Information provided by Buckeye Nutrition.
 585 ^b Gro 'N Win is a ration balancer pellet produced by Buckeye Nutrition.
 586 ^c Dyna K is a potassium chloride product made by The Mosaic Company.
 587 ^d Dynamate is a potassium and magnesium sulfate product made by The Mosaic Company.

588 **Table 3**
 589 Amino acid intakes (mg/kg body weight/day) for different dietary treatments and requirements based on the tissue
 590 composition of equine muscle for a controlled trial of yearling horses.
 591

	Dietary treatments						Standard deviation	Requirement estimates ^a
Lysine	76	90	104	118	127	136	7	113
Alanine	116	116	116	115	115	115	5	-
Arginine	116	115	115	115	114	114	8	84
Aspartate + Asparagine	138	136	134	132	131	130	12	-
Glutamate + Glutamine	365	367	368	370	371	373	19	-
Glycine	152	135	118	101	89	78	4	-
Histidine	25	25	25	25	25	25	3	73
Isoleucine	95	94	93	92	92	91	6	63
Leucine	195	196	196	196	196	196	8	123
Methionine	69	72	74	77	79	81	7	33
Phenylalanine	110	110	110	110	110	110	4	68
Proline	158	158	158	158	159	159	6	-
Serine	61	65	68	71	74	76	5	-
Threonine	66	66	66	66	66	66	3	70
Tyrosine	62	62	62	63	63	63	6	-
Valine	110	109	109	108	108	107	5	71

592 ^a Estimates based on gluteal muscle profiles as proposed by Bryden (1991).
 593

594 **Table 4**
 595 Plasma metabolites before (t = 0 min) feeding of the morning concentrate portion of diets providing varying lysine
 596 levels for a controlled trial of yearling horses.
 597

	Dietary lysine content (mg/kg body weight/day)						Pooled SE ^a	<i>P</i> values ^b		
	76	90	104	118	127	136		Treatment	Time	Treatment x Time
Urea (mmol/L)	4.4	4.6	4.3	4.1	3.5	4.8	1.5	0.72	<0.001	0.98
Glucose (mmol/L)	4.93	4.72	5.15	4.2	5.27	4.94	0.43	0.68	<0.001	0.44
Insulin (μIU/mL)	1.89	2.18	1.52	1.73	2.25	2.52	1.24	0.82	<0.001	0.84
Amino acids (μmol/L)										
Alanine	190	192	194	182	189	181	13	0.44	<0.001	0.52
Arginine	41	59	48	51	35	48	13	0.17	<0.001	0.56
Asparagine	19	19	20	17	18	19	4	0.67	<0.001	0.77
Aspartate	40	37	36	36	37	34	3	0.35	<0.001	0.8
Citrulline	104	106	107	105	100	107	11	0.84	<0.001	0.62
Glutamate	345	300	301	316	356	339	57	0.27	<0.001	0.89
Glutamine	223	275	264	230	227	219	42	0.42	<0.001	0.18
Glycine ^c	825	697	717	623	670	690	78	<0.001	<0.001	0.03
Histidine	70	73	72	68	66	74	7	0.83	<0.001	0.7
Isoleucine	57	59	56	57	53	56	8	0.46	<0.001	0.98
Leucine	105	107	105	102	98	100	16	0.4	<0.001	0.79
Lysine	95	106	102	110	105	118	19	<0.001	<0.001	<0.001
Methionine	80	78	76	78	81	71	16	0.2	<0.001	0.8
Ornithine	101	89	92	87	95	91	21	0.34	<0.001	0.87
Phenylalanine	62	63	63	61	61	64	6	0.59	<0.001	0.86
Proline	121	117	116	110	113	113	16	0.97	<0.001	0.88
Serine	271	265	268	241	247	251	28	0.32	<0.001	0.91
Taurine	51	44	49	51	50	51	5	0.75	<0.001	0.76
Threonine	137	132	133	119	123	139	14	0.32	<0.001	0.31
Tryptophan	7	6	6	6	6	6	1	0.73	0.03	0.55
Tyrosine	91	95	93	97	95	97	6	0.67	<0.001	0.97
Valine	152	146	140	141	131	140	18	0.25	<0.001	0.97

598 ^a SE, standard error.
 599 ^b Treatment, time and treatment by time interaction *P* values are shown in Tables 4 and 5.
 600 ^c Quadratic within baseline time point (*P* < 0.05).
 601

602 **Table 5**
 603 Plasma metabolites 90 min after feeding of the morning concentrate portion of diets providing varying lysine levels for
 604 a controlled trial of yearling horses.
 605

	Dietary lysine content (mg/kg body weight/day)						Pooled SE ^a	P values ^b		
	76	90	104	118	127	136		Treatment	Time	Treatment x Time
Urea (mmol/L)	13.7	13.6	13	13.6	13	11.9	1.5	0.72	<0.001	0.98
Glucose (mmol/L)	6.38	6.35	6.28	6.43	6.47	6.59	0.43	0.68	<0.001	0.44
Insulin (μIU/mL)	6.86	7.06	6.04	8.16	6.9	6.91	1.24	0.82	<0.001	0.84
Amino acids (μmol/L)										
Alanine	271	304	283	294	291	292	13	0.44	<0.001	0.52
Arginine	68	92	82	95	72	88	13	0.17	<0.001	0.56
Asparagine	39	34	35	37	33	35	4	0.67	<0.001	0.77
Aspartate	60	54	53	58	55	55	3	0.35	<0.001	0.80
Citrulline	141	135	137	132	129	131	11	0.84	<0.001	0.62
Glutamate	403	363	377	357	404	439	57	0.27	<0.001	0.89
Glutamine	445	451	454	497	445	418	42	0.42	<0.001	0.18
Glycine ^c	998	887	854	737	709	703	78	<0.001	<0.001	0.03
Histidine	97	101	99	97	103	100	7	0.83	<0.001	0.70
Isoleucine	80	82	78	85	77	81	8	0.46	<0.001	0.98
Leucine	170	164	163	180	160	174	16	0.40	<0.001	0.79
Lysine	148	181	199	286	288	323	19	<0.001	<0.001	<0.001
Methionine	215	215	189	232	210	203	16	0.20	<0.001	0.80
Ornithine	134	108	115	119	127	120	21	0.34	<0.001	0.87
Phenylalanine	85	86	84	90	86	89	6	0.59	<0.001	0.86
Proline	217	212	215	221	223	223	16	0.97	<0.001	0.88
Serine	350	323	337	314	319	315	28	0.32	<0.001	0.91
Taurine	64	63	59	60	62	61	5	0.75	<0.001	0.76
Threonine	221	192	193	203	205	196	14	0.32	<0.001	0.31
Tryptophan	7	7	7	7	6	7	1	0.73	0.03	0.55
Tyrosine	127	126	125	133	130	128	6	0.67	<0.001	0.97
Valine	199	190	182	195	177	191	18	0.25	<0.001	0.97

606 ^a SE, standard error.
 607 ^b Treatment, time and treatment by time interaction *P* values are shown in Tables 4 and 5.
 608 ^c Linear within 90 min time point (*P* < 0.05).
 609

610 **Table 6**
 611 Free amino acids (μmol/g) in freeze dried gluteus medius muscle from yearling horses fed varying levels of lysine ~100
 612 min after a morning concentrate meal.
 613

	Dietary lysine content (mg/kg body weight/day)						Pooled standard error	P values			
	76	90	104	118	127	136		Treatment	Linear	Quadratic	Cubic
3-Methyl histidine	0.86	0.66	0.81	1.03	0.99	1.04	0.16	0.29	0.08	0.44	0.20
Alanine	3.06	2.04	2.85	2.72	3.13	3.73	0.63	0.50	0.26	0.16	0.71
Asparagine	0.49	0.31	0.35	0.40	0.37	0.57	0.10	0.37	0.54	0.05	0.93
Aspartate	0.83	0.57	0.53	0.66	0.64	0.90	0.13	0.31	0.66	0.03	0.84
Arginine	0.26	0.25	0.25	0.25	0.25	0.27	0.27	0.06	0.82	0.01	0.53
Carnosine	86.42	71.64	85.90	94.23	93.47	91.31	13.68	0.72	0.34	0.76	0.27
Citrulline	0.40	0.27	0.38	0.36	0.36	0.40	0.07	0.63	0.71	0.36	0.45
Cysteine	8.06	6.25	6.31	7.05	7.27	7.00	1.86	0.96	0.90	0.50	0.54
Glutamate	4.75	3.09	4.19	4.10	4.46	4.57	0.83	0.67	0.69	0.30	0.34
Glutamine	5.61	3.42	4.42	4.32	4.54	6.60	1.14	0.33	0.46	0.04	0.96
Glycine	4.42	3.81	3.54	3.30	4.43	3.37	0.79	0.74	0.54	0.55	0.53
Histidine	0.40	0.23	0.33	0.27	0.31	0.31	0.08	0.54	0.60	0.29	0.39
Isoleucine	0.23	0.17	0.19	0.24	0.23	0.22	0.04	0.30	0.49	0.32	0.06
Leucine	0.52	0.38	0.44	0.50	0.51	0.51	0.08	0.51	0.47	0.33	0.17
Lysine	0.50	0.37	0.20	0.38	0.54	0.78	0.13	0.12	0.11	0.01	0.78
Methionine	0.54	0.45	0.46	0.56	0.51	0.55	0.07	0.80	0.63	0.44	0.42
Ornithine	1.02	0.39	1.01	0.77	0.64	0.79	0.39	0.48	0.79	0.67	0.42
Phenylalanine	0.10	0.09	0.11	0.09	0.10	0.09	0.02	0.98	0.79	0.97	0.73
Proline	0.52	0.35	0.44	0.44	0.45	0.53	0.09	0.74	0.75	0.24	0.60
Serine	1.39	0.86	1.04	1.13	1.10	1.29	0.18	0.33	0.93	0.07	0.29
Taurine	3.24	3.14	3.43	4.45	3.94	9.21	1.38	0.01	0.01	0.01	0.11
Threonine	1.25	0.86	0.86	1.05	1.54	1.44	0.45	0.61	0.38	0.19	0.53
Tryptophan	0.19	0.12	0.12	0.13	0.13	0.19	0.03	0.24	0.98	0.02	1.00
Tyrosine	0.41	0.29	0.39	0.42	0.45	0.39	0.06	0.21	0.34	0.65	0.04
Valine	0.51	0.38	0.42	0.48	0.46	0.46	0.07	0.61	0.94	0.35	0.18

Table 7
 Whole body phenylalanine kinetics in yearling Thoroughbred colts that were receiving graded levels of dietary lysine. ^a

	Graded lysine intakes (mg/kg body weight/day)						Pooled SE	P values			
	76	90	104	118	127	136		Treatment	Linear	Quadratic	Cubic
Phenylalanine flux (mmol/kg body weight/h)	49	49	52	52	46	49	3	0.53	0.76	0.26	0.97
Carbon dioxide production (mmol/kg body weight/h)	19411	18762	20794	20192	19883	17974	1085	0.22	0.71	0.06	0.13
Phenylalanine entering the free phenylalanine pool (mmol/kg body weight/h) ^b											
Phenylalanine from dietary intake ^c	13.5	13.9	13.8	13.6	13.4	13.5	0.2	0.22	0.27	0.11	0.06
Phenylalanine from protein breakdown	35.6	35.1	38.1	38.6	32.7	35.4	2.9	0.57	0.81	0.32	0.93
Phenylalanine leaving the free phenylalanine pool (mmol/kg body weight/h) ^b											
Phenylalanine oxidation	6.1	5.7	7.1	6.5	5.3	7.1	1.2	0.77	0.73	0.96	0.68
Non-oxidative phenylalanine disposal	42.7	43.1	44.5	45.4	40.8	41.6	2.8	0.81	0.70	0.31	0.87
Phenylalanine balance (mmol/kg body weight/h) ^d	7.4	8.2	6.7	7.0	8.2	6.4	1.3	0.79	0.66	0.85	0.89

^a Values are least squares means ± standard error (SE) determined using one-way analysis of variance.

^b Stochastic model of phenylalanine kinetics used: flux = rate of phenylalanine entry = rate of phenylalanine leaving; rate of phenylalanine entry = phenylalanine intake + phenylalanine release from protein breakdown; rate of phenylalanine leaving = phenylalanine oxidation + non-oxidative phenylalanine disposal; a splanchnic phenylalanine extraction rate of 50% was assumed when calculating the amount of dietary phenylalanine reaching general circulation.

^c Values were adjusted for estimated prececal digestibilities of 40 and 70% for forage and concentrate, respectively, and a splanchnic extraction of 26.5%.

^d Phenylalanine balance = non-oxidative phenylalanine disposal - phenylalanine release from protein breakdown.

626 **Figure legend**

627

628 Fig. 1. Plotting lysine intake and phenylalanine oxidation fail to yield a breakpoint to indicate the
629 lysine requirement for yearling Thoroughbred colts. Six colts were fed diets containing graded
630 amounts of lysine. Phenylalanine oxidation was not affected by diet ($P > 0.10$).

Figure 1